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THE SPECIFICITY OF UDP-GLUCOSE 4-EPIMERASE FROM THE YEAST *SACCHAROMYCES FRAGILIS**

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SUMMARY

1. The reactivity of UDP-glucose 4-epimerase (EC 5.1.3.2) from the yeast *Saccharomyces fragilis* toward 11 analogs of UDPG has been examined. The compounds tested involve stereochemical modifications of the hexosyl and ribosyl moieties of UDPG.

2. Of the 11 compounds only UDP-β-L-arabinose, UDP-D-fucose and UDP-D-xylose are epimerized. However, evidence is presented indicating that these epimerizations are due to contaminating enzymes and are not due to UDPG 4-epimerase.

3. The substances UDP-D-mannose, UDP-*N*-acetyl-D-galactosamine, UDP-D-allose and UDP-3-*O*-methyl-D-glucose were not epimerized. These observations are in agreement with the hypothesis of BUDOWSKY *et al.*[9] since the postulated hexose: uracil hydrogen bond, which is necessary for enzyme action in their mechanism, would be hindered in these substances.

4. dUDPG is not acted upon by the yeast epimerase. It has been reported to serve as a substrate for the epimerase from calf liver.

5. Other substrates which were not epimerized are UDP-β-D-glucose, UDP-D-glucuronic acid, UDP-4-*O*-methyl-D-glucose, ADPG, CDPG, GDPG, IDPG and dTDPG.

INTRODUCTION

The biological interconversion of glucose and galactose has been the object of considerable interest for many years. The reaction shown in Eqn. 1 was discovered

Abbreviations: UDPA, UDP-β-L-arabinose; UDPX, UDP-D-xylose; UDPF, UDP-D-fucose; UDPQ, UDP-D-quinovose.

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by LELOIR[1] and is catalyzed by UDP-glucose 4-epimerase (EC 5.1.3.2) hereafter called epimerase.

$$\text{UDPG} \rightleftarrows \text{UDPGal} \qquad (1)$$

The epimerase occurs widely in nature and has been studied from a variety of sources[2-4]. Although the enzymes differ according to source they all require NAD^+ for activity.

In earlier studies in this laboratory[5] it was observed that epimerase preparations from yeast *(Saccharomyces fragilis)* were capable of epimerizing UDP-β-L-arabinose (UDPA) and it had been reported by NEUFELD *et al.*[6] that yeast epimerase failed to act upon UDPA. It has also been reported that mung bean extracts contain UDPA-4-epimerase activity although it has not been separated from UDPG-4-epimerase activity or distinguished from it in any other way[6,7]. It is evident that, if the epimerization of UDPGal and UDPA are catalyzed by different enzymes, these enzymes should exhibit a strong specificity toward the groups at C-5 of the pyranose ring of the substrate, since UDPGal and UDPA differ only in substitution at C-5. Thus it was felt profitable to extend the work on the specificity pattern of the enzyme toward the pyranose moiety of the substrate and to determine whether or not the previous specificity results reported from this laboratory were influenced by the presence of more than one enzyme activity in the preparation used. In addition, such work would allow a further evaluation of the hypothesis[8,9] that UDPG 4-epimerase requires a specific secondary structure in its substrates.

METHODS AND MATERIALS

Enzymes

Yeast UDPG 4-epimerase was employed at three levels of purification. Prep. 1 and 2, purified by the procedure of MAXWELL AND DE ROBICHON-SZULMAJSTER[2], had specific activities of 262 and 620 mU/mg* of protein, respectively. Prep. 3, generously donated by Dr. H. KALCKAR, had a specific activity of 14 830 mU/mg of protein and migrated as a single component upon acrylamide gel electrophoresis. UDPG dehydrogenase (EC 1.1.1.22) had a specific activity of 202 mU/mg of protein after purification through Step 6 and assay according to STROMINGER *et al.*[10]. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and phosphoglucomutase (EC 2.7.5.1) were purchased from California Biochemicals Incorp.

Determination of specific activity of epimerase

Reactions were run in quartz cuvettes containing suitable enzyme concentrations, 0.1 μmole UDPGal, 1 μmole NAD^+, 10 mU of UDPG dehydrogenase and 150 μmoles of glycine buffer (pH 8.7) in a final volume of 0.5 ml. Reaction was followed by measuring NADH production at 24° in a Beckman DU spectrophotometer equipped with pinhole filter and photomultiplier. The rate of change of absorbance was kept between 0.01–0.04 per min and the change in absorbance was converted to μmoles of UDPG oxidized per min using an $E_{340\ m\mu}$ for NADH of $6.2 \cdot 10^3\ M^{-1} \cdot cm^{-1}$.

* The units are those recommended by the International Union of Biochemistry and are expressed in μmoles/min.

Preparation of carbohydrates

D-Allose was synthesized from a commercial preparation of D-ribose by cyanohydrin synthesis and subsequent reduction of D-α-allonolactone[11]. The yield of crystalline β-D-allose ($[\alpha]_D^{25} + 13°$ (*c* 1 in H_2O)) was 63% based on D-allonolactone and it had a melting point of 127–129°. The 3-*O*-methyl-D-glucose was prepared from 1,2:5,6-di-*O*-isopyropylidene-α-D-glucofuranose in 51% yield (m.p. 165.5–168°)[12]. The 4-*O*-methyl-D-glucose was obtained as an oil in 54% yield from 2,3:5,6-di-*O*-isopropylidene-D-glucose dimethyl acetal and characterized as its phenylosazone (m.p. 156–157°)[12,13]. The D-fucose obtained as a syrup was prepared in 80% yield by $LiAlH_4$ reduction of 1,2:3,4-di-*O*-isopropylidine-6-*O*-tosyl-D-galactose[14]. It was characterized by paper chromatography in Solvent A and found to contain only a trace of galactose. The D-[^{14}C]glucose was purchased from New England Nuclear Corp., Boston, Mass. D-Glucose, D-galactose, D-xylose, L-arabinose and D-mannose were all commercial preparations from Pfanstiehl Laboratories. Reference samples of 6-deoxy-D-glucose (quinovose), D-talose, 3-*O*-methyl-D-galactose, and 4-*O*-methyl-D-galactose, were supplied by Dr. B. A. LEWIS of this department.

Preparation of sugar acetates and sugar phosphates

All of the carbohydrate acetates were prepared by common laboratory procedures[15] and isolated in crystalline form where possible. The sugar phosphates were all prepared from their respective acetates by the MACDONALD method[16] except for the β-anomer of D-glucose 1-phosphate. It was prepared by previously described procedures[17–19], crystallized as its bis cyclohexylammonium salt, and recrystallized

TABLE I

PHYSICAL DATA ON SUGAR PHOSPHATES

Compound	*Sugar 1-phosphates salt*	*m.p.*	$[\alpha]_D^{25}$	
α-D-Allopyranose 1-phosphate	Bis cyclohexylammonium salt	179–180°	+26°	(*c* 2 in H_2O)
β-L-Arabinopyranose 1-phosphate	Bis cyclohexylammonium salt	187–188.5°	+90.6°	(*c* 2 in H_2O)
α-D-Galactopyranose 1-phosphate	Potassium	—	+91.5°	(*c* 0.5 in H_2O)
α-D-[^{14}C]Glucose 1-phosphate	Bis cyclohexylammonium salt*	166–170°d	+61°	(*c* 1.5 in H_2O)
β-D-Glucopyranose 1-phosphate	Bis cyclohexylammonium salt	165°d	+10°	(*c* 1 in H_2O)
α-D-3-*O*-Methyl-glucopyranose 1-phosphate	Brucine	—	+78.5°**	(*c* 2 in H_2O)
α-D-4-*O*-Methyl-glucopyranose 1-phosphate	Brucine	159–164°d	+ 6.3°	(*c* 5.3 in H_2O)
α-D-Fucopyranose 1-phosphate	Bis cyclohexylammonium salt	184.5–186°	+67.5°	(*c* 2 in H_2O)
α-D-Xylopyranose 1-phosphate	Bis cyclohexylammonium salt	152–158°	+56.8°	(*c* 2 in H_2O)
α-D-Mannopyranose 1-phosphate	Bis cyclohexylammonium salt	183–185°	+30.6°	(*c* 2 in H_2O)

* Recrystallized to a constant specific activity of 83 000 disint./min per μmole.
** Rotation taken with potassium salt since brucine salt has a negative rotation.

until free of α-D-glucose 1-phosphate as determined spectrophotometrically with phosphoglucomutase, glucose-6-phosphate dehydrogenase and NAD^+ (ref. 20).

All of the sugar phosphates were recrystallized 3 times to rid them of any possible contamination with the undesired anomer. This number of recrystallizations was found to be sufficient to obtain constant specific activity with α-D-[^{14}C]glucose 1-phosphate. They all had a ratio of hexose or pentose to orthophosphate of 1:1 after hydrolysis in 0.1 M HCl at 100° for 15 min. Physical data on the sugar phosphates are presented in Table I.

Preparation of nucleotides and nucleotide sugars

Nucleotide morpholidates were synthesized and analyzed as described by MOFFATT AND KHORANA[21]. dUMP was prepared from dCMP by oxidative deamination with $NaNO_2$ (ref. 22). The product was obtained as its calcium salt in 79% yield. The product was pure dUMP as determined by spectral analysis at pH's 2 and 12 and paper chromatography in Solvent D which clearly separates dCMP and dUMP.

Nucleoside monophosphates, NAD^+, $NADP^+$, dCMP and UDPG were purchased from P.L. Biochemicals Inc., Milwaukee, Wisc.

All chemically synthesized nucleotide sugars were prepared on a microscale by a modification of the procedure of ROSEMAN *et al.*[23], using a glass vacuum manifold system. Details of this procedure have been published elsewhere[24]. UDP-D-fucose (UDPF), UDPA and UDPGal were also synthesized on a large scale[23]. Analytical data for some nucleotide sugars is presented in Table II. UDPGal was assayed

TABLE II

ANALYTICAL DATA ON NUCLEOTIDE SUGARS

Compound	*Molar ratio*		
	Base	*Reducing sugar*	*Total phosphate*
UDP-D-fucose	1.00	0.96	1.97
UDP-β-L-arabinose	1.00	0.99	1.96
UDP-D-mannose	1.00	1.01	2.02
UDP-D-xylose	1.00	1.08	2.00
UDP-D-allose	1.00	1.02	2.02
UDP-β-D-glucose	1.00	0.99	2.02
UDP-3-*O*-methyl-D-glucose	1.00	0.98	2.04
UDP-4-*O*-methyl-D-glucose	1.00	1.02	2.08

enzymatically with epimerase and found to be 95% pure. UDP-D-[^{14}C]glucuronic acid was prepared in 97% yield by enzymic oxidation of UDP-D-[^{14}C]glucose with UDPG dehydrogenase[7]. UDP-D-[^{14}C]galactosamine (7.5 μC/μmole) was a gift from Dr. D. M. CARLSON, Western Reserve University.

Analytical methods

Reducing sugar was determined by either the PARK–JOHNSON[25] or NELSON–SOMOGYI[26] procedures. Protein was determined by a modified Folin–Lowry method[27] or a modified biuret procedure[28], and phosphorous by the method of FISKE AND

SUBBAROW[29]. ^{14}C determinations were made on liquid samples using a Nuclear Chicago scintillation spectrometer and on paper chromatograms by the use of a Nuclear Chicago 4 π radiochromatogram scanner. Paper chromatography of sugars, sugar phosphates, and nucleotide sugars was performed on Whatman No. 1 paper using the following solvents (all concentrations v/v): Solvent A, pyridine–ethyl acetate–water 2:5:7 (upper phase) (ref. 30); Solvent B, 1-butanol–pyridine–water 6:4:3 (ref. 31); Solvent C, 1-butanol–ethanol–water 40:11:19 (ref. 31); Solvent D, absolute ethanol–1 M ammonium acetate (pH 7.5) 75:25 (ref. 32); Solvent E, absolute ethanol–methylethylketone–0.5 M morpholinium tetraborate (pH 8.6) 7:2:3 (ref. 33); and Solvent F, 1-butanol–acetic acid–water 2:1:1 (ref. 34). Ultraviolet-absorbing compounds were detected on paper with a Mineralight hand lamp, Model 3L 2537. Free sugars were detected by the method of TREVELYAN, PROCTER AND HARRISON[35]. Sugar phosphates were detected using the procedure of HANES AND ISHERWOOD[36] using an ultraviolet light to develop the color[37].

Incubation of nucleotide sugar analogs with epimerase

The nucleotide sugars were incubated for 8 h at 37° with 282 mU of enzyme and 240 μmoles of glycine buffer (pH 8.7) in a total volume of 1.0 ml. The amount of substrate incubated with the enzyme varied from 0.5 μmole of the non-radioactive analogs to 10 000 counts/min of the radioactive analogs. A boiled enzyme control was run with every incubation. After incubation, 1.0 ml of 1 M HCl was added to the reaction mixture which was then hydrolyzed for 10 min at 100°. After cooling, the solution was deionized by passage through columns of IR-120 (H^+) and IR-45 (OH^-). It was then streaked on Whatman No. 1 paper and chromatographed with a solvent chosen for its ability to separate the particular reactant sugar from its 4-epimer.

An alternate method was used to determine the extent of reaction with some of the analogs. The reaction was stopped by heating at 100° for 45 sec and the reaction mixture then streaked on washed Whatman 3-mm paper and chromatographed using Solvent D. After chromatography the nucleotide sugar band was cut out and eluted with water. The eluant was concentrated, streaked on Whatman No. 1 paper, and chromatographed in Solvent E. This system will separate UDPF from UDP-D-quinovose (UDPQ), UDPG from UDPGal, and UDP-D-xylose (UDPX) from UDPA. The UDP-sugar bands were eluted and their concentration determined by reading the absorbance of the eluant, against water, at 262 mμ. A correction for contaminating ultraviolet-absorbing material from the paper and the solvent can be applied by subtracting the absorbance obtained at 318 mμ. This is possible since the absorbance of the contaminants at 318 mμ is the same as their absorbance at 262 mμ while uridine has no absorbance at 318 mμ.

RESULTS

Reactivity of nucleotide sugars with epimerase

Among the substrate analogs tested with epimerase preparations only UDPX, UDPA and UDPF were acted upon. D-Fucose, D-fucose 1-phosphate, L-arabinose, β-L-arabinose 1-phosphate and xylose were not epimerized. D-Xylose 1-phosphate showed slight reaction with epimerase Prep. 2, but no reaction with epimerase Prep. 3.

TABLE III

REACTIVITY OF SUBSTRATES WITH EPIMERASE

See METHODS for incubation of nucleotide sugar analogs with epimerase; the alternate method was used.

Substrate	*Molar ratio after incubation with epimerase*	*Equilibrium ratio*
UDPG	$\frac{\text{UDPG}}{\text{UDPGal}} = 2.82 \pm .20$	3.0 (ref. 1)
UDPX	$\frac{\text{UDPX}}{\text{UDPA}} = 1.09 \pm .08$	1.0 (ref. 7)
UDPA	$\frac{\text{UDPA}}{\text{UDPX}} = 1.26 \pm .09$	1.0 (ref. 7)
UDPF	$\frac{\text{UDPF}}{\text{UDPQ}} = 1.62 \pm .14$	—*

* The equilibrium ratio of the UDPF–UDPQ interconversion has not been determined.

UDPF was epimerized to a new nucleotide sugar upon incubation with all three epimerase preparations. This new ultraviolet-absorbing compound was separated from UDPF by paper chromatography in Solvent E, $R_{UDPF} = 1.2$. It had a uridine spectrum and when subjected to mild acid hydrolysis released a sugar with a mobility identical to 6-deoxy-D-glucose (quinovose) in Solvents A, B, and C. Similar experi-

TABLE IV

NUCLEOTIDE SUGARS TESTED WITH EPIMERASE

Modification of UDPG or UDPGal: H, hexosyl moiety; C-number, position of modification in the sugar ring; C, chemical modification; S, stereochemical modification; R, ribosyl moiety.

Compound	*Modification*
UDP-D-glucose	Natural substrate
UDP-D-galactose	Natural substrate
UDP-D-xylose*	H, C-5, C
UDP-β-L-arabinose*	H, C-5, C
UDP-D-fucose*	H, C-5, C
UDP-D-[^{14}C]glucuronic acid	H, C-5, C
UDP-4-*O*-methyl-D-glucose	H, C-4, C
UDP-3-*O*-methyl-D-glucose	H, C-3, C
UDP-D-allose	H, C-3, S
UDP-D-mannose	H, C-2, S
UDP-*N*-acetyl-D-[^{14}C]galactosamine	H, C-2, C
UDP-β-D-glucose	H, C-1, S
dUDP-D-[^{14}C]glucose	R, C-2, C
ADP [^{14}C]glucose	Base
CDP [^{14}C]glucose	Base
GDP [^{14}C]glucose	Base
IDP [^{14}C]glucose	Base
dTDP [^{14}C]glucose	Base

* These substrates were epimerized, but by contaminating enzymes. None of the other substances, with the exception of UDPG and UDPGal, were acted upon.

ments using Solvents A, B, and F demonstrated arabinose in the hydrolyzate of the product from the incubation of UDPX with epimerase and xylose in the hydrolyzate of the product from the incubation of UDPA.

UDPF, UDPX, and UDPA failed to inhibit the conversion of UDPGal to UDPG at concentrations up to 200 times that of UDPGal. Inhibition would certainly be expected if these substances were epimerized by the same enzyme that acts upon UDPGal. In order to investigate this matter further, the K_m for UDPF was determined and found to be $2.5 \cdot 10^{-4}$ M. The magnitude of this Michaelis constant, in comparison with that for UDPGal ($1.86 \cdot 10^{-5}$ M) leaves no doubt whatever that inhibition should have been observed at the 200-fold concentration of UDPF. In view of this it was assumed that the epimerization of the three substances was catalyzed by enzymes other than UDPG 4-epimerase.

More evidence in support of this idea was gained by investigating the activity of UDPG 4-epimerase preparations at different stages of purification. A crude preparation (Prep. 1) readily catalyzed the epimerization of UDPG, UDPX, UDPA and UDPF in the standard 8-h incubation period (Table III). When UDPX and

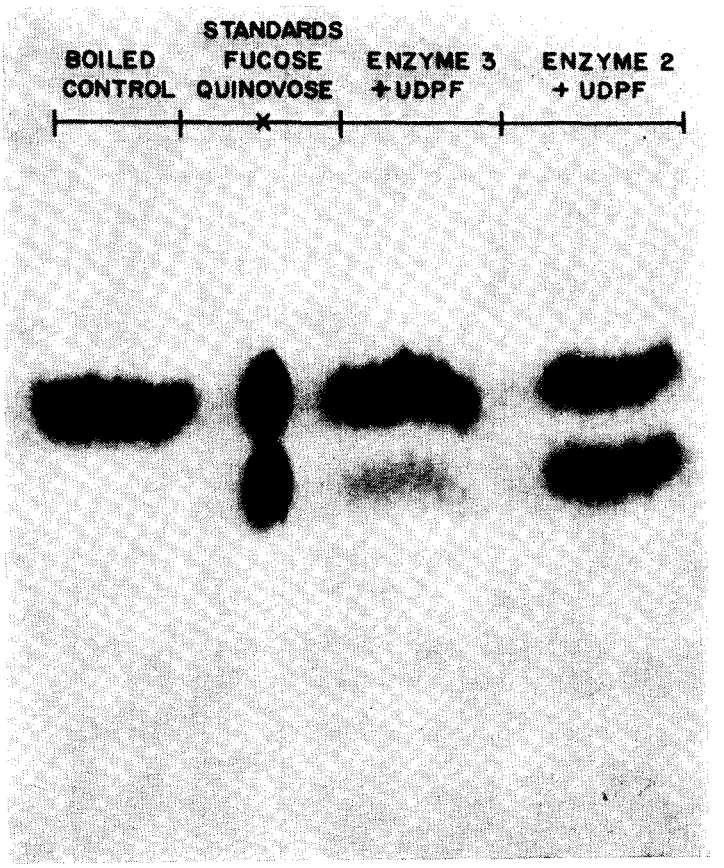

Fig. 1. The effect of degree of purification on the ability of a UDPG-4-epimerase preparation to catalyze the epimerization of UDPF. The time of incubation was 8 h.

UDPA were incubated with Prep. 2 or 3, only traces of the epimerized product could be detected by paper chromatography of the free sugars after hydrolysis. The epimerization of UDPF was also very much reduced upon purification of the enzyme as seen in Fig. 1.

It is therefore concluded that the epimerization of UDPX, UDPA, and UDPF is the result of contaminating epimerase activities in even the purest available preparations of UDPG 4-epimerase. A list of the substrate analogs, together with the nature of the modification of their structures from that of the natural substrates, is presented in Table IV.

DISCUSSION

UDPG 4-epimerase from yeast appears to have a strict specificity for the hexosyl moiety of its substrate. Three substrate analogs were epimerized; however, the evidence suggests that these three analogs, UDPF, UDPX and UDPA, were epimerized by enzymes other than UDPG 4-epimerase. This being so, the epimerase has a strict specificity for the hydroxymethyl group at C-5 of the pyranose ring. It is also sensitive to structural or stereochemical modifications at C-1, C-2, and C-3, and to structural modification at C-4.

dUDPG was not epimerized by the yeast enzyme although it has been reported[8,9] that calf-liver epimerase will epimerize this molecule. Thus, the specificity of yeast epimerase is slightly different from that of calf-liver epimerase. This difference may be because yeast epimerase has either a more stringent requirement for intramolecular hydrogen bonding in the substrate, or it requires a binding of the 2′ hydroxyl group to some specific site on the enzyme.

dTDPG was not epimerized but the lack of reactivity could be due entirely to the lack of the 2′ hydroxyl group. In order to determine if thymine will substitute for uracil, TDPG must be made.

None of the base substitutions for uracil resulted in molecules which were reactive with the yeast epimerase. It has been suggested that the base moiety is important for enzyme specificity in reactions involving nucleotide sugars[38], *i.e.* a sort of handle. However, from the work reported here it is obvious that the sugar moiety is also very important. Calf-liver epimerase can accommodate several modifications of the base moiety without complete loss of reactivity[8,9]. These studies led to a proposed secondary structure for the substrate. The secondary structure hypothesis has the merit that it offers an explanation for specificities involving the entire substrate molecule and not just isolated non-interacting moieties.

Four substrates which would have hindered hexose:uracil hydrogen bonding of the type described by BUDOWSKY *et al.*[9] failed to react with epimerase. These were UDP-D-allose, UDP-3-*O*-methyl-D-glucose, UDP-D-mannose, and UDP-*N*-acetyl-D-galactosamine. Models of UDP-β-D-glucose would not readily form the proposed secondary structure. These observations tend to support the secondary structure hypothesis and also explain why the hexosyl moiety is very important in determining specificity. A secondary structure similar to that of BUDOWSKY *et al.* was proposed earlier[39]. It was pointed out that a knowledge of the three dimensional structure of UDPG and UDPGal would be of great value in interpreting the mechanism of action of the epimerase.

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